CA

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 April 2000 (06.04.2000)

PCT

(10) International Publication Number
WO 00/018881 A3

(51) International Patent Classification[7]: C07K 19/00, C12N 9/10, 15/62, C12P 21/02, 21/04

(21) International Application Number: PCT/US99/22776

(22) International Filing Date:
30 September 1999 (30.09.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/102,413 30 September 1998 (30.09.1998) US

(71) Applicant *(for all designated States except US)*: NEW ENGLAND BIOLABS, INC. [US/US]; 32 Tozer Road, Beverly, MA 01915 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: XU, Ming-Qun [US/CN]; 40 Crescent Road, Hamilton, MA 01982 (US). EVANS, Thomas, C. [US/US]; 1st floor, 68 Albion Street, Somerville, MA 02143 (US).

(74) Agent: WILLIAMS, Gregory, D.; New England Biolabs, Inc., 32 Tozer Road, Beverly, MA 01915 (US).

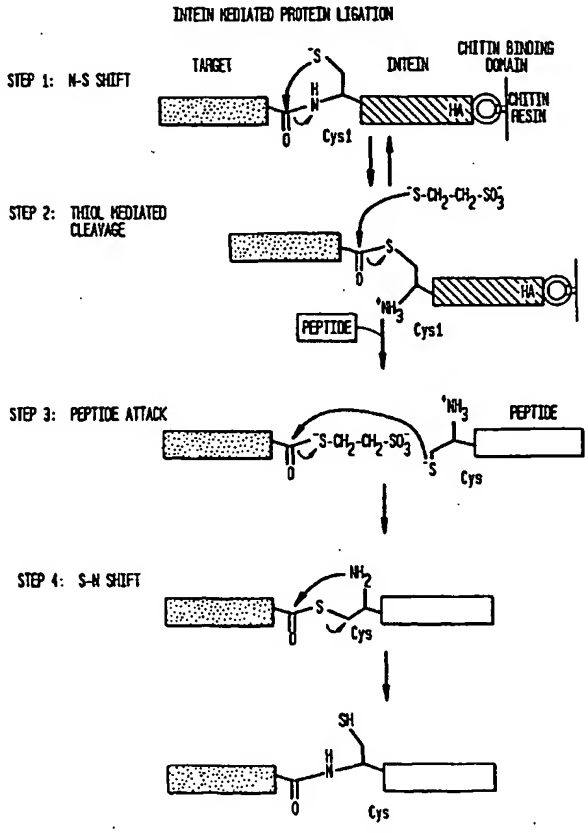
(81) Designated States *(national)*: CA, JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *with international search report*

[Continued on next page]

(54) Title: INTEIN MEDIATED PEPTIDE LIGATION



(57) Abstract: An in vitro method for producing a semi-synthetic fusion protein is provided, whereby a target protein fused to an intein - a protein splicing element - is selectively cleaved in a first step as depicted in Figure 1 with a thiol reagent, forming a carboxyl-terminal thioester of the target protein and releasing the target protein from the intein. In a subsequent step as shown in Figure 1, a desired, synthetic, protein or peptide having an amino-terminal cysteine is ligated to the target protein. Standard thiol-reagents such as DTT, or thiol-reagents optimized for ligation such as the odorless MESNA, may be used in the first step. The method permits the direct ligation of a desired peptide to a thioester bond that had linked a target protein to an intein. An in vivo variation of the method will permit production of a cytotoxic protein: a truncated, inactive, form of the protein fused to an intein is introduced in vivo, this fusion product is then selectively cleaved, and a synthetic protein or peptide is subsequently ligated at a carboxyl-terminal thioester of the target protein in order to restore the native activity of the cytotoxic protein.

WO 00/018881 A3

(88) Date of publication of the international search report:
23 November 2000

(48) Date of publication of this corrected version:
22 August 2002

(15) Information about Corrections:
see PCT Gazette No. 34/2002 of 22 August 2002, Section II

Previous Corrections:
see PCT Gazette No. 15/2001 of 12 April 2001, Section II
see PCT Gazette No. 40/2000 of 5 October 2000, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTEIN MEDIATED PEPTIDE LIGATION

BACKGROUND OF THE INVENTION

Genetic engineering is a powerful approach to the manipulation of proteins. However, genetic methodologies are constrained by the use of only naturally coded amino acids. Furthermore, cytotoxic proteins are difficult to obtain by expression and isolation from a living source, since the expression of the toxic protein can result in death of the host.

To some extent, protocols have been developed to circumvent these problems, for example, total chemical synthesis (Kent, S. B. (1988) *Ann. Rev. Biochem.* 57:957-989), use of misacylated tRNAs (Noren, et al., (1989) *Science* 244:182-188), and semi-synthetic techniques (reviewed in Offord, R. (1987) *Protein Eng.* 1:151-157; Roy. et al. (1994) *Methods in Enzymol.* 231:194-215; Wallace, C. J. (1993) *FASEB* 7:505-515). However, all of these procedures are limited by either the size of the fragment which can be generated or by low reaction yield.

It would therefore be desirable to develop a high-yield, semi-synthetic technique to allow *in vitro* fusion of a synthetic protein or peptide fragment to an expressed protein without limitation as to the size of the fused fragments.

Likewise, in order to produce cytotoxic proteins, it would be desirable to develop a method of fusing a synthetic fragment, *in vitro*, to an inactive, expressed protein, so as to restore protein activity post-production from the host.

The modified Sce VMA intein has been used to generate thioester-tagged proteins for use in ligation (Example 19, U.S.S.N. 08/811,492, filed June 16, 1997; Chong, (1996) *J. Biol. Chem.*, 271(36):22159-22168; Chong, (1997) *Gene*, 192:271-281; and Muir, et al. (1998) *Proc. Natl. Acad. Sci* USA 95:6705-6710).

Some disadvantages have been low yields due to poor cleavage of the Sce VMA intein with thiol-reagents that are optimum for ligation, the need for large peptide quantities due to on-column reactions, the use of odoriferous reagents, and/or low protein yields due to the use of a large, eukaryotic intein.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a method for producing a semi-synthetic fusion protein *in vitro*, comprising the steps of producing a target protein fused to a protein splicing element (an intein) and selectively cleaving the fusion and ligating a synthetic

protein or peptide at the C-terminal thioester of the target protein, which overcome many of the disadvantages and problems noted above. Specifically, the present invention has higher yields due to better thiol-induced cleavage with thiol reagents which have been optimized for the ligation reaction. Off-column ligation allows for sample concentration as well as the use of less peptide. In a particularly preferred embodiment, thiol reagents such as 2-mercaptoethanesulfonic acid (MESNA), which is an odorless thiol-reagent, is used for cleavage and ligation along with the Mxe intein, which is from a bacterial source and often expresses better in bacterial cells. Furthermore, the present invention allows peptides to be directly ligated to the thioester bond formed between an intein and the target protein. The present invention also provides a method for producing a cytotoxic protein, comprising the steps of producing a truncated, inactive form of the protein *in vivo* which is fused to a protein splicing element, and selectively cleaving the fusion and ligating a synthetic protein or peptide at a C-terminal thioester of the target protein to restore the activity of the native cytotoxic protein. Recombinant vectors for producing such cleavable fusion proteins are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow diagram depicting the chemical reactions which enable intein-mediated peptide ligation. The

thioester generated at the C-terminus of the target protein during IMPACT™ purification was used in a 'native chemical ligation' reaction. This allowed the ligation of a synthetic peptide to a bacterially expressed protein. A typical ligation reaction involved the expression of the target protein-intein-CBD fusion followed by binding to a chitin resin. A thiol reagent induced cleavage of the intein. The target was eluted from the chitin resin and a synthetic peptide was added. The ligation reaction proceeded overnight.

Figure 2 is a gel depicting the results of cleavage and ligation reactions using various thiols. Cleavage and ligation reactions with different thiols visualized on 10-20% Tricine gels. MYB (a fusion protein of maltose binding protein-Sce VMA intein (N454A)-chitin binding domain) and MXB (a fusion protein of maltose binding protein-Mxe GyrA (N198A) intein-chitin binding domain) were incubated overnight at 4°C with various thiols (50 mM) in 150 mM Tris, 100 mM NaCl, pH 8 in the presence of a 30 amino acid peptide with an N-terminal cysteine. The peptide ligates to the C-terminus of MBP. Lanes 1-5 ligation with MYB. Lane 1 no thiol. Lane 2 dithiothreitol. Lane 3 2-mercaptoethanesulfonic acid. Lane 4 3-mercaptopropionic acid. Lane 5 thiophenol. Lanes 6-10 ligation with MXB. Lane 6 no thiol. Lane 7 dithiothreitol. Lane 8 2-mercaptoethanesulfonic acid. Lane 9 3-mercaptopropionic acid. Lane 10 thiophenol.

Figure 3 is a gel depicting direct ligation of a peptide to the thioester formed between the Sce VMA intein and maltose binding protein. SDS-PAGE of direct ligation reaction with a 10-20% Tricine gel. Lane 1: a precursor protein (MYBleu) consisting of maltose binding protein-Sce VMA1 intein-chitin binding domain was heated to >95°C for 5 minutes in a buffer of 50 mM Trizma base, pH 8.5 containing 100 mM NaCl, 1% SDS, and mM tris-(2-carboxyethyl)phosphine (TCEP) followed by overnight incubation at room temperature. The precursor (MYBleu) is visible along with the Sce VMA1 intein (Y) and maltose binding protein (M), which are cleavage products. Lane 2: the precursor protein was subjected to the same conditions as described in Lane 1 except that the 30 amino acid peptide (1 mM) was added. The precursor (MYB) and cleavage products (Y and M) are visible along with the ligation product (M+30mer) formed when the 30 amino acid peptide fuses to maltose binding protein.

Figure 4 is a diagram depicting the pTXB1 expression vector of Example I (SEQ ID NO:7 and SEQ ID NO:8).

Figure 5 is the DNA sequence of pTXB1 (SEQ ID NO:5).

Figure 6 is a gel depicting the results of the *Hpa*I protein ligation reaction. Protein ligation reactions examined on 10-20% Tricine gels. Lane 1: clarified cells extract after IPTG (0.5 mM) induction of ER2566 cells containing the pTXB2-*Hpa*I

plasmid. The fusion protein of *HpaI*$_{223}$-Mxe GyrA-intein-CBD (52 kDa) is visible. Lane 2: cell extract as in Lane 1 after passage over a chitin column, which results in the binding of the fusion protein. Lane 3: *HpaI*$_{223}$ (25.7 kDa) after cleavage from the fusion protein by addition of MESNA. Lane 4: ligation product of *HpaI*$_{223}$ (0.2 mg/mL) with 1 mM of a 31 amino acid peptide (ligation product 29.6 kDa), representing the residues necessary to generate full length *Hpa*I, after overnight incubation at 4°C. Lane 5: full length *Hpa*I from a recombinant source (29.6 kDa) containing BSA (66 kDa) and two impurities.

Figure 7 is a western blot of various proteins ligated to a biotinylated peptide. Proteins purified with the Mxe GyrA IMPACT™ derivative were ligated to a synthetic peptide which contained an antibody recognition sequence.

DETAILED DESCRIPTION OF THE INVENTION

The ligation methods of the present invention are based on the discovery that a cysteine or peptide fragment containing an N-terminal cysteine may be fused, *in vitro*, to a bacterially expressed protein produced by thiol-induced cleavage of an intein (U.S. Patent No. 5,496,714; Example 19 of U.S.S.N. 08/811,492 filed June 16, 1997; Chong, et al., (1996) *supra* and Chong, et al., (1997) *supra*.

The ligation procedure disclosed herein utilizes a protein splicing element, an intein (Perler, et al., (1994) *Nucleic Acids Res.* 22:1125-1127) to precisely create a thioester at the C-terminal α-carbon of an expressed protein. This reactive thioester could be present between the target protein and intein or generated by the addition of a thiol reagent. Previously the generation such a thioester was described using an intein (CIVPS) that was modified to undergo thiol inducible cleavage at its N-terminal junction in the presence of thiol reagent dithiothreitol (DTT) (Chong, et al. (1997) *supra*; Comb, et.al. U.S. Patent No. 5,496,714). This C-terminal thioester was previously used in a 'native chemical ligation' type reaction (Dawson, et al., (1994) *Science* 266:776-779) to fuse ^{35}S-cysteine or a peptide fragment containing an N-terminal cysteine to a bacterially expressed protein (Example 19, Comb, et.al. U.S. Patent No. 5,834,247, Chong (1996) *supra* and Chong (1997) *supra*.

The ligation method of the instant invention begins with the purification of the thioester-tagged target protein using an intein as described (Chong, et.al. (1997) *supra*). The direct ligation method of the instant invention begins with the isolation of a precursor composed of the target protein-intein-CBD. In one preferred embodiment, the host cell is bacterial. In other embodiments the host cell may be yeast, insect, or mammalian. A cysteine thiol at the N-terminus of a synthetic peptide nucleophilicly attacks a thioester present

on the freshly isolated C-terminal α-carbon of the target protein or directly attacks the thioester present between the target protein and intein. This initially generates a thioester between the two reactants which spontaneously rearranges into a native peptide bond (Figure 1).

In order to optimize the ligation efficiency so that greater than 90% of the bacterially expressed target protein can be fused to the synthetic peptide or protein, specific thiol reagents and inteins are screened. In a preferred embodiment, the intein may be any CIVPS, such as *Sce* VMA, *Mxe* GyrA or derivatives of mutants thereof, and the thiol reagent is 2-mercapto-ethanesulfonic acid, thiophenol, DTT, or 3-mercaptopropionic acid (Comb, et al., U.S. Patent No. 5,496,714; U.S. Patent No. 5,834,247).

In one particularly preferred embodiment, an intein whose protein splicing activity has been blocked by mutation is utilized. The mutant must, however, retain the ability to undergo the N-S shift, thus allowing thioester formation between itself and an N-terminal protein. This thioester can then be nucleophilicly attacked by a thiol reagent or by the N-terminal cysteine of a peptide sequence. For example, by mutating the C-terminal asparagine (asn 198) of an intein from the GyrA gene of *Mycobacterium xenopi* (Telenti, et al., (1997) *J Bacteriol* 179:6378-6382) to an alanine created a

thiol inducible cleavage element. This modified intein cleaved well with thiol reagents that were optimal for the ligation reaction, such as MESNA and thiophenol. Furthermore, optimal thiol reagent and intein combinations can be determined by incubating a precursor protein containing the intein of interest with a wide variety of thiol reagents followed by determination of the extent of cleavage of the precursor protein (Figure 2).

The use of such intein and specific thiol reagents leads to optimal yields and high ligation efficiencies; typically greater than 90% of the N-terminal ligation fragment can be modified.

The ligation methods of the present invention expand the ability to incorporate non-coded amino acids into large protein sequences by generating a synthetic peptide fragment with fluorescent probes, spin labels, affinity tags, radiolabels, or antigenic determinants and ligating this to an *in vivo* expresed protein isolated using a modified intein.

Furthermore, this procedure allows the isolation of cytotoxic proteins by purifying an inactive truncated precursor from a host source, for example bacteria, and generating an active protein or enzyme after the ligation of a synthetic peptide. For example, restriction endonucleases which have not successfully been cloned by traditional

methods may be produced in accordance with the present invention.

Also, the direct ligation procedure allows the ligation of a protein or peptide sequence to another protein or peptide sequence without the use of exogenous thiol reagents. Direct ligation relies on the nucleophilic attack of the N-terminal amino acid of one peptide on the thioester formed between a target protein and an intein (Figure 3).

In summary, a fusion protein can be created using the methods of the present invention that possesses unique properties which, currently, can not be generated genetically.

The Examples presented below are only intended as specific preferred embodiments of the present invention and are not intended to limit the scope of the invention. The present invention encompasses modifications and variations of the methods taught herein which would be obvious to one of ordinary skill in the art.

The references cited above and below are herein incorporated by reference.

EXAMPLE I

Creation of vectors pTXB1 and pTXB2 for ligation:

Asparagine 198 of the *Mxe* GyrA intein (Telenti, et al., (1997) *J Bacteriol.* 179:6378-6382) was mutated to alanine by linker insertion into the *Xmn*I and *Pst*I sites of pmxeMIPTyrXmnSPdel to create pMXP1. The *Xmn*I site was originally introduced into the unmodified *Mxe* GyrA intein sequence by silent mutagenesis. The *Pst*I site was a unique site in the plasmid. The linker was composed of mxe#3 (5'-GGTTCGTCAGCCACGCTACTGGCCTCACCGGTTGATAGCTGCA-3') (SEQ ID NO:1) and mxe#4 (5'-GCTATCAACCGGTGAGGCCAGTAG CGTGGCTGACGAACC-3') (SEQ ID NO:2).

Into pMXP1 another linker composed of mxe#1 (5'-TC GAATCTAGACATATGGCCATGGGTGGCGGCCGCCTCGAGGGCTCTTCC TGCATCACGGGAGATGCA-3') (SEQ ID NO:3) and mxe#2 (5'-CTAG TGCATCTCCCGTGATGCAGGAAGAGCCCTCGAGGCGHGCCGCCACCCA TGGCCATATGTCTAGAT-3') (SEQ ID NO:4) was inserted into the *Xho*I and *Spe*I sites to introduce a multiple cloning site (*Xba*I-*Nde*I-*Nco*I-*Not*I-*Xho*I-*Sap*I) before the *Mxe* GyrA intein (pMXP2).

The 0.6 kilobase *Not*I to *Age*I fragment of pMXP2 was ligated into the same sites in pTYB1 (IMPACT kit, New England Biolabs, Beverly, MA) and the *Nco*I to *Age*I fragment of pMXP2

was cloned into pTYB3 (IMPACT kit, New England Biolabs, Beverly, MA) to create plasmids pTXB1 (see Figure 4 and 5) (SEQ ID NO:5) and pTXB2, respectively. These vectors have a multiple cloning site upstream of the modified *Mxe* GyrA intein-chitin binding domain fusion. This allows the insertion of a target gene of interest inframe with the intein and chitin binding domain (CBD).

Creation of vectors pMYBleu for ligation:

pMYBleu was as described in Chong, et al., (1998), *J. Biol. Chem.* 273:10567-10577. This vector consisted of maltose binding protein upstream of the Sce VMA intein-chitin binding domain. A leucine is present at the -1 position instead of the native residue (which is a glycine).

Purification of Thioester-Tagged Proteins:

Protein purification was as described using the *Sce* VMA intein (Chong, et.al., (1997) *Gene* 192:271-281) with slight modification. ER2566 cells (IMPACT T7 instruction manual from New England Biolabs, Beverly, MA) containing the pTXB vector with the appropriate insert were grown to an OD_{600} of 0.5-0.6 at 37°C at which point they were induced with 0.5 mM IPTG overnight at 15°C. Cells were harvested by centrifugation and lysed by sonication (performed on ice). The

three part fusion protein was bound to chitin beads (10 mL bed volume, Figure 6, lanes 1 and 2) equilibrated in Buffer A (50 mM Tris, pH 7.4, and 500 mM NaCl), and washed with 10 column volumes of Buffer A to remove unbound material.

Cleavage was initiated using a buffer of 50 mM 2-mercaptoethanesulfonic acid (MESNA), 50 mM Tris, pH 8.0 and 100 mM NaCl. Other thiol reagents were also used at other times, such as thiophenol, dithiothreitol, and/or 3-mercaptopropionic acid. After overnight incubation at from 4-25°C protein was eluted from the column (Figure 6 lane 3). This protein contained a thioester at the C-terminus.

Purification of MYB. MYBleu and MXB:

Full length precursor proteins consisting of maltose binding protein-*Sce* VMA intein (N454A)-chitin binding domain (MYB) and maltose binding protein-*Mxe* GyrA (N198A) intein-chitin binding domain (MXB) were purified after induction and sonication, as described above, by applying the sonicated sample to a 10 mL column of amylose resin (New England Biolabs, Beverly, MA). Unbound proteins were washed from the column with 10 column volumes of Buffer A (see purification of thioester-tagged proteins). Bound proteins were eluted with a buffer of 50 mM Tris, pH 8, containing 100 mM NaCl and 10 mM maltose. Fractions were collected and protein

concentrations were determined using the Bio-Rad Protein Assay (Hercules, CA).

Peptide Synthesis:

Peptides for subsequent ligation reactions were synthesized on an ABI model 433A peptide synthesizer utilizing *FastMoc*™ chemistry (Fields, et al., (1991) *Pept Res* 4, 95-101) at a 0.085 mmol scale. Preloaded HMP (p-hydroxymethylphenoxymethyl) polystyrene resins (Applied Biosystems, Foster City, CA) functionalized at 0.5 mmol/g was used in conjunction with Fmoc/NMP chemistry utilizing HBTU amino acid activation (Dourtoglou, et al., (1984) *Synthesis* 572-574; Knorr, et al., (1989) *Tetrahedron Lett* 30, 1927-1930). Fmoc amino acids were purchased from Applied Biosystems (Foster City, CA).

Synthesis proceeded with a single coupling during each cycle. Peptide cleavage from the resin and simultaneous removal of side chain protecting groups was facilitated by the addition of cleavage mixture (Perkin Elmer, Norwalk, CT) consisting of 0.75 g phenol, 0.25 mL 1,2-ethanedithiol, 0.5 mL deionized H_2O, and 10 mL TFA. The resin was flushed with nitrogen and gently stirred at room temperature for 3 hours. Following filtration and precipitation into cold (0°C) methyl-t-butyl ether, the precipitate in the ether fraction was

collected by centrifugation. The peptide precipitate was vacuum dried and analyzed by mass spectrometry using a Perceptive Biosystems (Framingham, MA) MALDI-TOF mass spectrometer.

Final purification was by HPLC using a Waters HPLC system with a Lambda-Max Model 481 Multiwavelength detector (set at 214 nm), 500 series pumps and automated gradient controller with a Vydac semi-preparative C18 column. Elution of the peptide was with a 60 minute linear gradient of 6-60% acetonitrile (v/v) in an aqueous solution of 0.1% TFA (v/v).

Protein Cleavage and Ligation Reactions:

Cleavage of MYB and MXB: The precursor protein (1 mg/mL) was incubated overnight at 4°C with or without a thiol reagent (50 mM) in 150 mM Tris, pH 8, containing 100 mM NaCl.

Ligation reactions with MYB and MXB: The precursor protein (1 mg/mL) was treated as described for cleavage except that a 30 amino acid peptide (1 mM final concentration, NH_2-CAYKTTQANKHIIVACEGNPYVPVHFDASV-COOH (SEQ ID NO:6) was also included in the reaction (Figure 2).

Ligation reactions after purification of thioester-tagged proteins: Lyophilized peptides (New England Biolabs, Beverly, MA) were added (to 1 mM final concentration) directly to the thioester-tagged protein freshly isolated from the chitin column. The reaction was allowed to proceed overnight at from 4-25°C. In both ligation procedures the condensation of the reactants is visible on a 10-20% Tricine gel (Figure 6). The ligation reaction was tested in conditions of 5-150 mM Tris or HEPES buffers, 50-1000 mM NaCl, 10 mM Maltose, and pH 6-11 and 0-6 M Urea.

Direct Ligation Reactions:

MYBleu (1 mg/mL) was incubated in 6 M Urea or 1% SDS, pH 7.5-8.5, 50-200 mM NaCl, and 1 mM of a 30 amino acid peptide (NH_2CAYKTTQANKHIVVACEGNPYVPVHFDASV-COOH (SEQ ID NO:6)). The MYBleu was incubated for 0-180 minutes at either 4°C or 100°C prior to the addition of the 30 amino acid peptide. Ligation reactions proceeded overnight at either 4°C or 25°C.

EXAMPLE II

Labeling a target protein: Maltose Binding Protein

Maltose binding protein (MBP, 42 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

A biotinylated peptide possessing an N-terminal cysteine (CDPEK*DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the ε-amino group of the lysine residue) was ligated to the freshly purified target protein as described above. Briefly, 4 μL of biotinylated peptide (10 mM) were mixed with a 36 μL aliquot of the freshly purified MBP sample. The mixture was incubated at 4°C overnight.

Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

EXAMPLE III

Labeling a target protein: Bst DNA Polymerase I Large Fragment (Bst Pol 1)

Bst DNA Polymerase I large fragment (67 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

A biotinylated peptide possessing an N-terminal cysteine (CDPEK*DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the ε-amino group of the lysine residue) was ligated to the freshly purified target protein as described. Briefly, 4 μL of biotinylated peptide (10 mM) were mixed with a 36 μL aliquot of the freshly purified Bst Pol 1 sample. The mixture was incubated at 4°C overnight.

Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

EXAMPLE IV

Labeling a target protein: Paramyosin

Paramyosin (29 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

A biotinylated peptide possessing an N-terminal cysteine (CDPEK*DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the ε-amino group of the lysine residue) was ligated to the freshly purified target protein as described. Briefly, 4 μL of biotinylated peptide (10 mM) were mixed with a 36 μL aliquot of the freshly purified paramyosin sample. The mixture was incubated at 4°C overnight.

Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

EXAMPLE V

Labeling a target protein: *E. coli* Thioredoxin

E. coli thioredoxin (12 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

A biotinylated peptide possessing an N-terminal cysteine (CDPEK*DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the ε-amino group of the lysine residue) was ligated to the freshly purified target protein as described. Briefly, 4 μL of biotinylated peptide (10 mM) were mixed with a 36 μL aliquot of the freshly purified thioredoxin sample. The mixture was incubated at 4°C overnight.

Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

EXAMPLE VI

Isolation of a cytotoxic protein:

The ligation procedure of Example I was applied to the isolation of a potentially cytotoxic protein. An endonuclease from *Haemophilus parainfluenzae* (*Hpa*I; Ito, et al., (1992) *Nucleic Acids Res* 20:705-709) was generated by ligating an inactive truncated form of the enzyme expressed in *E. coli* (ER2566 cells, New England Biolabs, Inc., Beverly, MA) with the missing amino acids that were synthesized chemically.

The first 223 amino acids of *Hpa*I (full length *Hpa*I is 254 amino acids) were fused in frame with the modified *Mxe* GyrA intein and the CBD. The 223 amino acid *Hpa*I fragment was isolated as described for purification of thioester tagged proteins. The truncated *Hpa*I displayed no detectable enzymatic activity.

A synthetic peptide representing the 31 amino acids needed to complete *Hpa*I was ligated onto the 223 amino acid truncated form of *Hpa*I by the method of Example I.

Enzymatic Assay for *Hpa*I:

The activity of the fused *Hpa*I was determined by its ability to digest Lambda DNA (New England Biolabs, Beverly, MA). Serial dilutions of ligated or truncated *Hpa*I, with the appropriate peptide added to 1 mM, were incubated with 1 μg of Lambda DNA for 1 hour at 37°C in a buffer of 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol, and 170 μg/mL BSA (total volume 30 μL). Digestion reactions were visualized on 1% agarose gels permeated with ethidium bromide. One unit of *Hpa* I was defined as the amount of enzyme necessary to digest 1 μg of Lambda DNA in one hour at 37°C.

The newly ligated *Hpa*I had a specific activity of 0.5-1.5x10^6 units/mg which correlated well with the expected value of 1-2x10^6 units/mg for the full length enzyme.

WHAT IS CLAIMED IS:

1. A method for fusing an expressed protein with a peptide, said method comprising the steps of:
   (a) generating at least one C-terminal thioester-tagged target protein;
   (b) generating at least one target peptide having a specified N-terminal; and
   (c) ligating said target peptide to said target protein.

2. The method of claim 1, wherein said target protein is generated from a first plasmid comprising an intein having N-terminal cleavage activity.

3. The method of claim 2, wherein said intein comprises an intein having a cysteine residue at the N-terminal of the intein.

4. The method of claim 3, wherein said target protein is generated by thiol reagent-induced cleavage of said intein.

5. The method of claim 4, wherein said thiol reagent is selected from the group consisting of MESNA, thiophenol, DTT, ß-mercaptoethanol or derivatives thereof.

6. A fusion protein produced by the method of any one of claims 1-5.

7. A cyclic protein produced by the method of claim 1.

8. A modified intein comprising a mutant Mxe GyrA intein capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of an adjacent target protein.

9. A method of generating a reactive thioester comprising contacting a thiol reagent selected from the group consisting essentially of MESNA, thiophenol, DTT, ß-mercaptoethanol or derivatives thereof with a precursor comprising a target protein and intein.

10. A method for screening thiol reagents which cleave a target intein comprising the steps of:

(a) isolating a precursor comprising a protein and a modified intein;

(b) contacting a thiol reagent with the precursor of step (a);

(c) determining whether a splicing or cleaving event occurs.

11. The method of claim 10, comprising the further step of determining whether the spliced or cleaved product of step (c) can ligate to a target peptide having an N-temrinal cytokine.

FIG. 1

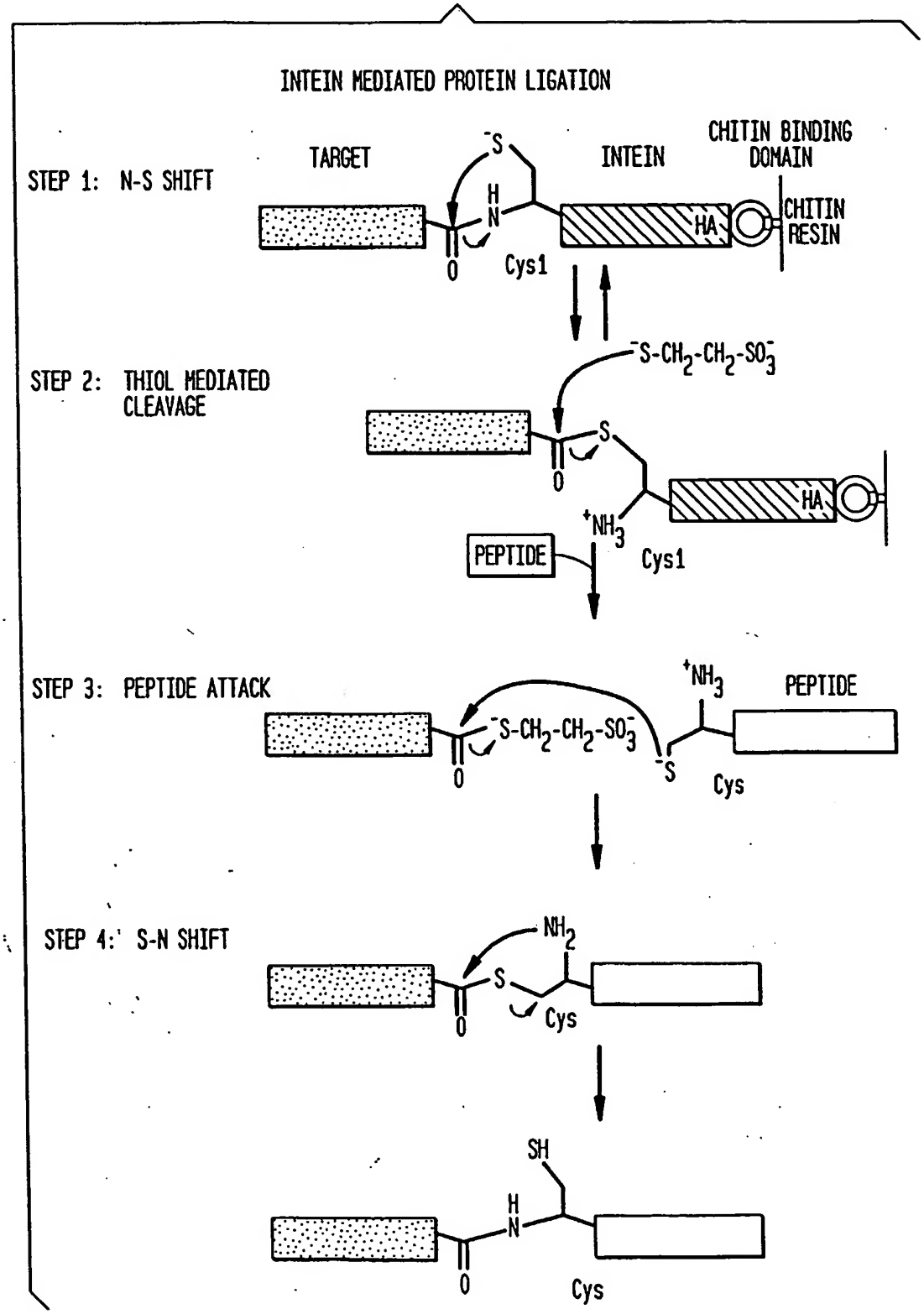

INTEIN MEDIATED PROTEIN LIGATION
STEP 1: N-S SHIFT
TARGET
INTEIN
CHITIN BINDING DOMAIN
CHITIN RESIN
HA
Cys1
STEP 2: THIOL MEDIATED CLEAVAGE
PEPTIDE
Cys1
STEP 3: PEPTIDE ATTACK
PEPTIDE
Cys
STEP 4: S-N SHIFT
Cys
Cys

FIG. 2

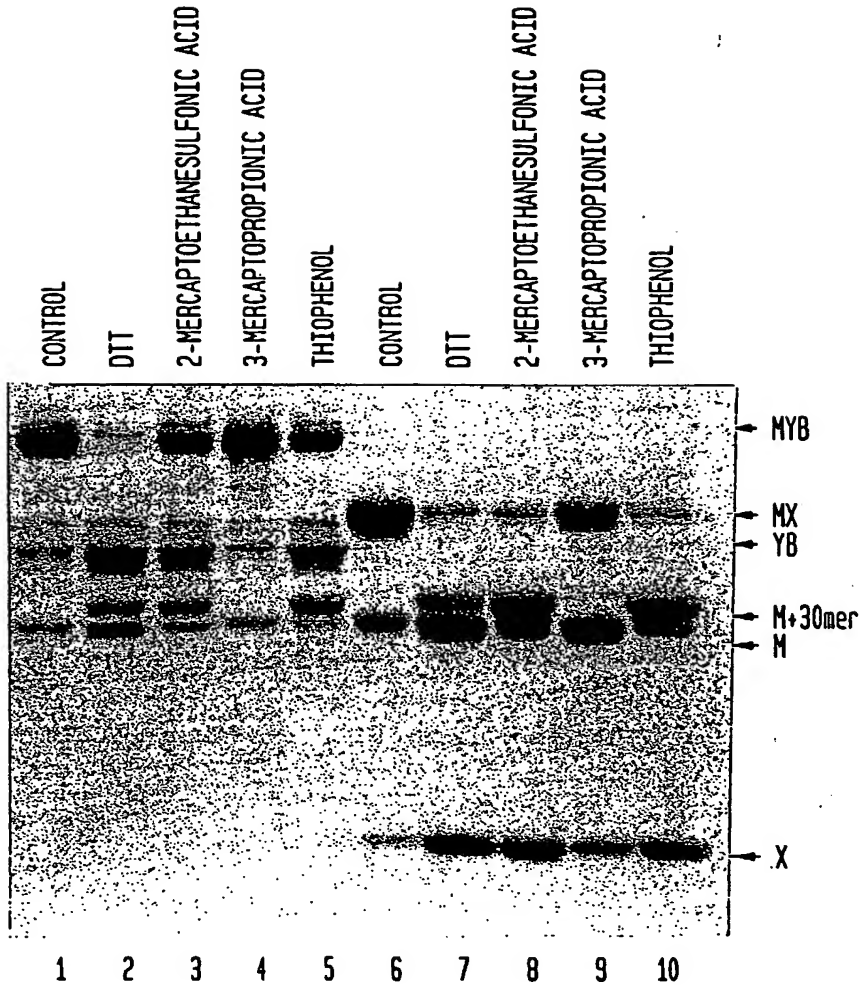


FIG. 3

DIRECT LIGATION REACTON

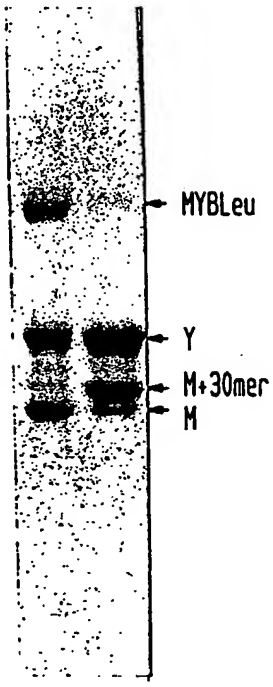
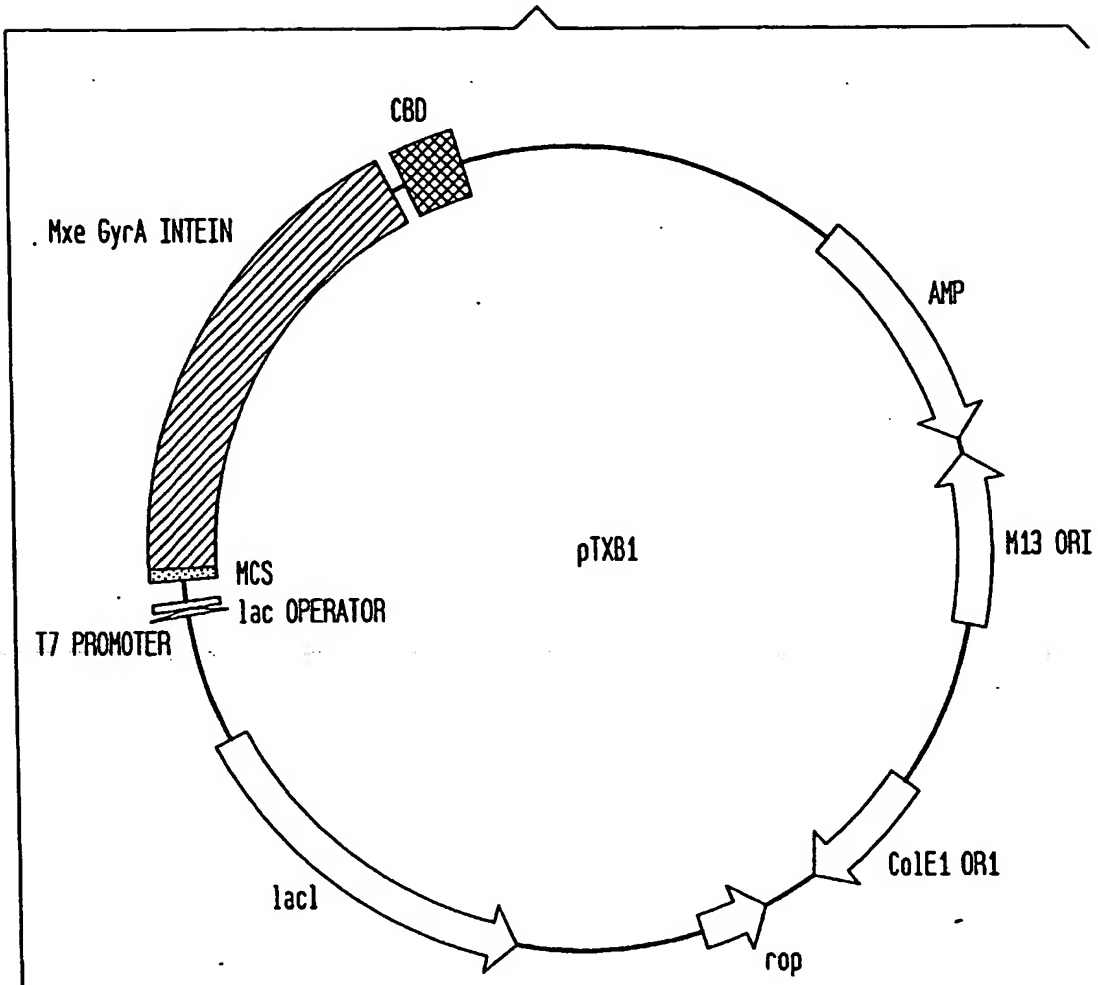


FIG. 4



pTXB1
....Met Ala Met Gly Gly Gly Arg Leu Glu Gly Ser Ser Cysl...Intein
CAT ATG GCC ATG GGT GGC GGC CGC CTC GAG GGC TCT TCC TGC
NdeI NcoI NotI XhoI SapI

FIG. 5A

DNA Sequence of pTXB1 plasmid

140- 997	beta-lactamase (Ap)
1042-1555	M13 origin
2254	ColE1 origin
2626-2814	rop
3376-4455	lacIq
5440-5456	T7 promoter
5440-5459	T7 universal primer (forward)
5457	first nucleotide of the T7 transcript
5459-5483	lac operator
5513-5519	Shine-Dalgarno sequence (T7 gene 10)
5525-5572	Multiple cloning site
5573-6166	Mxe GyrA intein (N198A)
6197-6352	Chitin-binding domain
6375-6497	T7 transcription terminator

TXB1.seq.old Length: 6503 March 17, 1998 11:14 Type: N
Check: 1445 ..

```
   1  AACTACGTCA GGTGGCACTT TTCGGGGAAA TGTGCGCGGA ACCCCTATTT
  51  GTTTATTTTT CTAAATACAT TCAAATATGT ATCCGCTCAT GAGACAATAA
 101  CCCTGATAAA TGCTTCAATA ATATTGAAAA AGGAAGAGTA TGAGTATTCA
 151  ACATTTCCGT GTCGCCCTTA TTCCCTTTTT TGCGGCATTT TGCCTTCCTG
 201  TTTTTGCTCA CCCAGAAACG CTGGTGAAAG TAAAAGATGC TGAAGATCAG
 251  TTGGGTGCAC GAGTGGGTTA CATCGAACTG GATCTCAACA GCGGTAAGAT
 301  CCTTGAGAGT TTTCGCCCCG AAGAACGTTC TCCAATGATG AGCACTTTTA
 351  AAGTTCTGCT ATGTGGCGCG GTATTATCCC GTGTTGACGC CGGGCAAGAG
 401  CAACTCGGTC GCCGCATACA CTATTCTCAG AATGACTTGG TTGAGTACTC
 451  ACCAGTCACA GAAAAGCATC TTACGGATGG CATGACAGTA AGAGAATTAT
 501  GCAGTGCTGC CATAACCATG AGTGATAACA CTGCGGCCAA CTTACTTCTG
 551  ACAACGATCG GAGGACCGAA GGAGCTAACC GCTTTTTTGC ACAACATGGG
 601  GGATCATGTA ACTCGCCTTG ATCGTTGGGA ACCGGAGCTG AATGAAGCCA
 651  TACCAAACGA CGAGCGTGAC ACCACGATGC CTGTAGCAAT GGCAACAACG
 701  TTGCGCAAAC TATTAACTGG CGAACTACTT ACTCTAGCTT CCCGGCAACA
 751  ATTAATAGAC TGGATGGAGG CGGATAAAGT TGCAGGACCA CTTCTGCGCT
 801  CGGCCCTTCC GGCTGGCTGG TTTATTGCTG ATAAATCTGG AGCCGGTGAG
 851  CGTGGGTCTC GCGGTATCAT TGCAGCACTG GGGCCAGATG GTAAGCCCTC
```

FIG. 5B

```
 901  CCGTATCGTA GTTATCTACA CGACGGGGAG TCAGGCAACT ATGGATGAAC
 951  GAAATAGACA GATCGCTGAG ATAGGTGCCT CACTGATTAA GCATTGGTAA
1001  CTGTCAGACC AAGTTTACTC ATATATACTT TAGATTGATT TACCCCGGTT
1051  GATAATCAGA AAAGCCCCAA AAACAGGAAG ATTGTATAAG CAAATATTTA
1101  AATTGTAAAC GTTAATATTT TGTTAAAATT CGCGTTAAAT TTTTGTTAAA
1151  TCAGCTCATT TTTTAACCAA TAGGCCGAAA TCGGCAAAAT CCCTTATAAA
1201  TCAAAAGAAT AGCCCGAGAT AGGGTTGAGT GTTGTTCCAG TTTGGAACAA
1251  GAGTCCACTA TTAAAGAACG TGGACTCCAA CGTCAAAGGG CGAAAAACCG
1301  TCTATCAGGG CGATGGCCCA CTACGTGAAC CATCACCCAA ATCAAGTTTT
1351  TTGGGGTCGA GGTGCCGTAA AGCACTAAAT CGGAACCCTA AAGGGAGCCC
1401  CCGATTTAGA GCTTGACGGG GAAAGCCGGC GAACGTGGCG AGAAAGGAAG
1451  GGAAGAAAGC GAAAGGAGCG GGCGCTAGGG CGCTGGCAAG TGTAGCGGTC
1501  ACGCTGCGCG TAACCACCAC ACCCGCCGCG CTTAATGCGC CGCTACAGGG
1551  CGCGTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT CATGACCAAA
1601  ATCCCTTAAC GTGAGTTTTC GTTCCACTGA GCGTCAGACC CCGTAGAAAA
1651  GATCAAAGGA TCTTCTTGAG ATCCTTTTTT TCTGCGCGTA ATCTGCTGCT
1701  TGCAAACAAA AAAACCACCG CTACCAGCGG TGGTTTGTTT GCCGGATCAA
1751  GAGCTACCAA CTCTTTTTCC GAAGGTAACT GGCTTCAGCA GAGCGCAGAT
1801  ACCAAATACT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC CACTTCAAGA
1851  ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT GTTACCAGTG
1901  GCTGCTGCCA GTGGCGATAA GTCGTGTCTT ACCGGGTTGG ACTCAAGACG
1951  ATAGTTACCG GATAAGGCGC AGCGGTCGGG CTGAACGGGG GGTTCGTGCA
2001  CACAGCCCAG CTTGGAGCGA ACGACCTACA CCGAACTGAG ATACCTACAG
2051  CGTGAGCTAT GAGAAAGCGC CACGCTTCCC GAAGGGAGAA AGGCGGACAG
2101  GTATCCGGTA AGCGGCAGGG TCGGAACAGG AGAGCGCACG AGGGAGCTTC
2151  CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT TCGCCACCTC
2201  TGACTTGAGC GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATG
2251  GAAAAACGCC AGCAACGCGG CCTTTTTACG GTTCCTGGCC TTTTGCTGGC
2301  CTTTTGCTCA CATGTTCTTT CCTGCGTTAT CCCCTGATTC TGTGGATAAC
```

FIG. 5C

2351 CGTATTACCG CCTTTGAGTG AGCTGATACC GCTCGCCGCA GCCGAACGAC

2401 CGAGCGCAGC GAGTCAGTGA GCGAGGAAGC TATGGTGCAC TCTCAGTACA

2451 ATCTGCTCTG ATGCCGCATA GTTAAGCCAG TATACACTCC GCTATCGCTA

2501 CGTGACTGGG TCATGGCTGC GCCCCGACAC CCGCCAACAC CCGCTGACGC

2551 GCCCTGACGG GCTTGTCTGC TCCCGGCATC CGCTTACAGA CAAGCTGTGA

2601 CCGTCTCCGG GAGCTGCATG TGTCAGAGGT TTTCACCGTC ATCACCGAAA

2651 CGCGCGAGGC AGCTGCGGTA AAGCTCATCA GCGTGGTCGT GCAGCGATTC

2701 ACAGATGTCT GCCTGTTCAT CCGCGTCCAG CTCGTTGAGT TTCTCCAGAA

2751 GCGTTAATGT CTGGCTTCTG ATAAAGCGGG CCATGTTAAG GGCGGTTTTT

2801 TCCTGTTTGG TCACTTGATG CCTCCGTGTA AGGGGGAATT TCTGTTCATG

2851 GGGGTAATGA TACCGATGAA ACGAGAGAGG ATGCTCACGA TACGGGTTAC

2901 TGATGATGAA CATGCCCGGT TACTGGAACG TTGTGAGGGT AAACAACTGG

2951 CGGTATGGAT GCGGCGGGAC CAGAGAAAAA TCACTCAGGG TCAATGCCag

3001 ccgaACGCCA GCAAGACGTA GCCCAGCGCG TCGGCCGCCA TGCCGGCGAT

3051 AATGGCCTGC TTCTCGCCGA AACGTTTGGT GGCGGGACCA GTGACGAAGG

3101 CTTGAGCGAG GGCGTGCAAG ATTCCGAATA CCGCAAGCGA CAGGCCGATC

3151 ATCGTCGCGC TCCAGCGAAA GCGGTCCTCG CCGAAAATGA CCCAGAGCGC

3201 TGCCGGCACC TGTCCTACGA GTTGCATGAT AAAGAAGACA GTCATAAGTG

3251 CGGCGACGAT AGTCATGCCC CGCGCCCACC GGAAGGAGCT GACTGGGTTG

3301 AAGGCTCTCA AGGGCATCGG TCGAGATCCC GGTGCCTAAT GAGTGAGCTA

3351 ACTTACATTA ATTGCGTTGC GCTCACTGCC CGCTTTCCAG TCGGGAAACC

3401 TGTCGTGCCA GCTGCATTAA TGAATCGGCC AACGCGCGGG GAGAGGCGGT

3451 TTGCGTATTG GGCGCCAGGG TGGTTTTTCT TTTCACCAGT GAGACGGGCA

3501 ACAGCTGATT GCCCTTCACC GCCTGGCCCT GAGAGAGTTG CAGCAAGCGG

3551 TCCACGCTGG TTTGCCCCAG CAGGCGAAAA TCCTGTTTGA TGGTGGTTAA

3601 CGGCGGGATA TAACATGAGC TGTCTTCGGT ATCGTCGTAT CCCACTACCG

3651 AGATATCCGC ACCAACGCGC AGCCCGGACT CGGTAATGGC GCGCATTGCG

3701 CCCAGCGCCA TCTGATCGTT GGCAACCAGC ATCGCAGTGG GAACGATGCC

3751 CTCATTCAGC ATTTGCATGG TTTGTTGAAA ACCGGACATG GCACTCCAGT

FIG. 5D

```
3801  CGCCTTCCCG TTCCGCTATC GGCTGAATTT GATTGCGAGT GAGATATTTA
3851  TGCCAGCCAG CCAGACGCAG ACGCGCCGAG ACAGAACTTA ATGGGCCCGC
3901  TAACAGCGCG ATTTGCTGGT GACCCAATGC GACCAGATGC TCCACGCCCA
3951  GTCGCGTACC GTCTTCATGG GAGAAAATAA TACTGTTGAT GGGTGTCTGG
4001  TCAGAGACAT CAAGAAATAA CGCCGGAACA TTAGTGCAGG CAGCTTCCAC
4051  AGCAATGGCA TCCTGGTCAT CCAGCGGATA GTTAATGATC AGCCCACTGA
4101  CGCGTTGCGC GAGAAGATTG TGCACCGCCG CTTTACAGGC TTCGACGCCG
4151  CTTCGTTCTA CCATCGACAC CACCACGCTG GCACCCAGTT GATCGGCGCG
4201  AGATTTAATC GCCGCGACAA TTTGCGACGG CGCGTGCAGG GCCAGACTGG
4251  AGGTGGCAAC GCCAATCAGC AACGACTGTT TGCCCGCCAG TTGTTGTGCC
4301  ACGCGGTTGG GAATGTAATT CAGCTCCGCC ATCGCCGCTT CCACTTTTTC
4351  CCGCGTTTTC GCAGAAACGT GGCTGGCCTG GTTCACCACG CGGGAAACGG
4401  TCTGATAAGA GACACCGGCA TACTCTGCGA CATCGTATAA CGTTACTGGT
4451  TTCACATTCA CCACCCTGAA TTGACTCTCT TCCGGGCGCT ATCATGCCAT
4501  ACCGCGAAAG GTTTTGCGCC ATTCGATGGT GTCCCGGATC TCGACGCTCT
4551  CCCTTATGCG ACTCCTGCAT TAGGAAGCAG CCCAGTAGTA GGTTGAGGCC
4601  GTTGAGCACC GCCGCCGCAA GGAATGGTGC ATGCCGCCCT TCCGTCTTCA
4651  AGAATTAATT CCCAATTCCA GGCATCAAAT AAAACGAAAG GCTCAGTCGA
4701  AAGACTGGGC CTTTCGTTTT ATCTGTTGTT TGTCGGTGAA CGCTCTCCTG
4751  AGTAGGACAA ATCCGCCGGG AGCGGATTTG AACGTTGCGA AGCAACGGCC
4801  CGGAGGGTGG CGGGCAGGAC GCCCGCCATA AACTGCCAGG AATTAATTCC
4851  AGGCATCAAA TAAAACGAAA GGCTCAGTCG AAAGACTGGG CCTTTCGTTT
4901  TATCTGTTGT TTGTCGGTGA ACGCTCTCCT GAGTAGGACA AATCCGCCGG
4951  GAGCGGATTT GAACGTTGCG AAGCAACGGC CCGGAGGGTG GCGGGCAGGA
5001  CGCCCGCCAT AAACTGCCAG GAATTAATTC CAGGCATCAA ATAAAACGAA
5051  AGGCTCAGTC GAAAGACTGG GCCTTTCGTT TTATCTGTTG TTTGTCGGTG
5101  AACGCTCTCC TGAGTAGGAC AAATCCGCCG GGAGCGGATT TGAACGTTGC
5151  GAAGCAACGG CCCGGAGGGT GGCGGGCAGG ACGCCCGCCA TAAACTGCCA
5201  GGAATTAATT CCAGGCATCA AATAAAACGA AAGGCTCAGT CGAAAGACTG
```

FIG. 5E

5251 GGCCTTTCGT TTTATCTGTT GTTTGTCGGT GAACGCTCTC CTGAGTAGGA

5301 CAAATCCGCC GGGAGCGGAT TTGAACGTTG CGAAGCAACG GCCCGGAGGG

5351 TGGCGGGCAG GACGCCCGCC ATAAACTGCC AGGAATTGGG GATCGGAATT

5401 AATTCCCGGT TTAAACCGGG GATCTCGATC CCGCGAAATT AATACGACTC

5451 ACTATAGGGG AATTGTGAGC GGATAACAAT TCCCCTCTAG AAATAATTTT

5501 GTTTAACTTT AAGAAGGAGA TATAcatatg gctagctcgc gagtcgacgg

5551 cggccgcctc gagggctctt ccTGCATCAC GGGAGATGCA CTAGTTGCCC

5601 TACCCGAGGG CGAGTCGGTA CGCATCGCCG ACATCGTGCC GGGTGCGCGG

5651 CCCAACAGTG ACAACGCCAT CGACCTGAAA GTCCTTGACC GGCATGGCAA

5701 TCCCGTGCTC GCCGACCGGC TGTTCCACTC CGGCGAGCAT CCGGTGTACA

5751 CGGTGCGTAC GGTCGAAGGT CTGCGTGTGA CGGGCACCGC GAACCACCCG

5801 TTGTTGTGTT TGGTCGACGT CGCCGGGGTG CCGACCCTGC TGTGGAAGCT

5851 GATCGACGAA ATCAAGCCGG GCGATTACGC GGTGATTCAA CGCAGCGCAT

5901 TCAGCGTCGA CTGTGCAGGT TTTGCCCGCG GAAAACCCGA ATTTGCGCCC

5951 ACAACCTACA CAGTCGGCGT CCCTGGACTG GTGCGTTTCT TGGAAGCACA

6001 CCACCGAGAC CCGGACGCCC AAGCTATCGC CGACGAGCTG ACCGACGGGC

6051 GGTTCTACTA CGCGAAAGTC GCCAGTGTCA CCGACGCCGG CGTGCAGCCG

6101 GTGTATAGCC TTCGTGTCGA CACGGCAGAC CACGCGTTTA TCACGAACGG

6151 GTTCGTCAGC CACGCTACTG GCCTCACCGG TCTGAACTCA GGCCTCACGA

6201 CAAATCCTGG TGTATCCGCT TGGCAGGTCA ACACAGCTTA TACTGCGGGA

6251 CAATTGGTCA CATATAACGG CAAGACGTAT AAATGTTTGC AGCCCCACAC

6301 CTCCTTGGCA GGATGGGAAC CATCCAACGT TCCTGCCTTG TGGCAGCTTC

6351 AATGActgca ggaaggGGAT CCGGCTGCTA ACAAAGCCCG AAAGGAAGCT

6401 GAGTTGGCTG CTGCCACCGC TGAGCAATAA CTAGCATAAC CCCTTGGGGC

6451 CTCTAAACGG GTCTTGAGGG GTTTTTTGCT GAAAGGAGGA ACTATATCCG

6501 GAT

FIG. 6

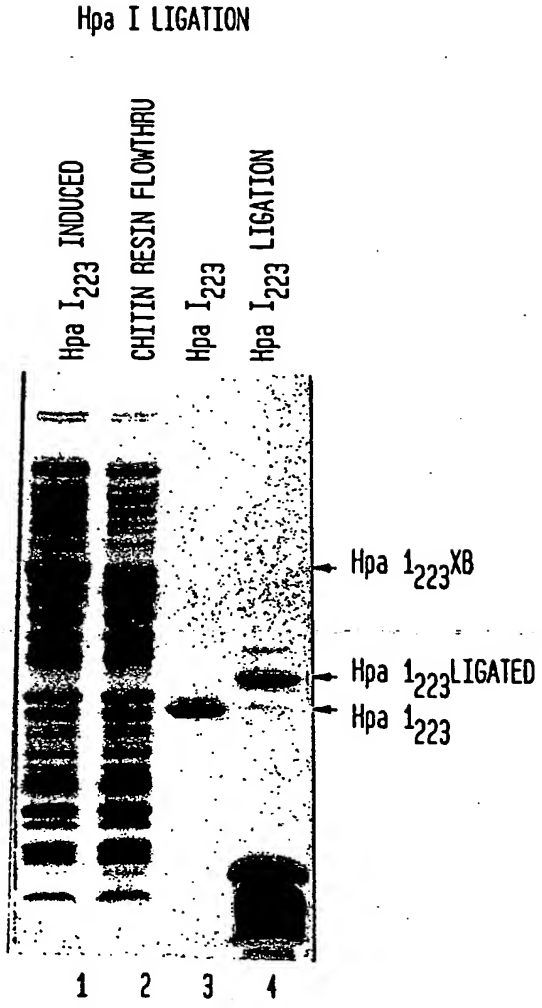
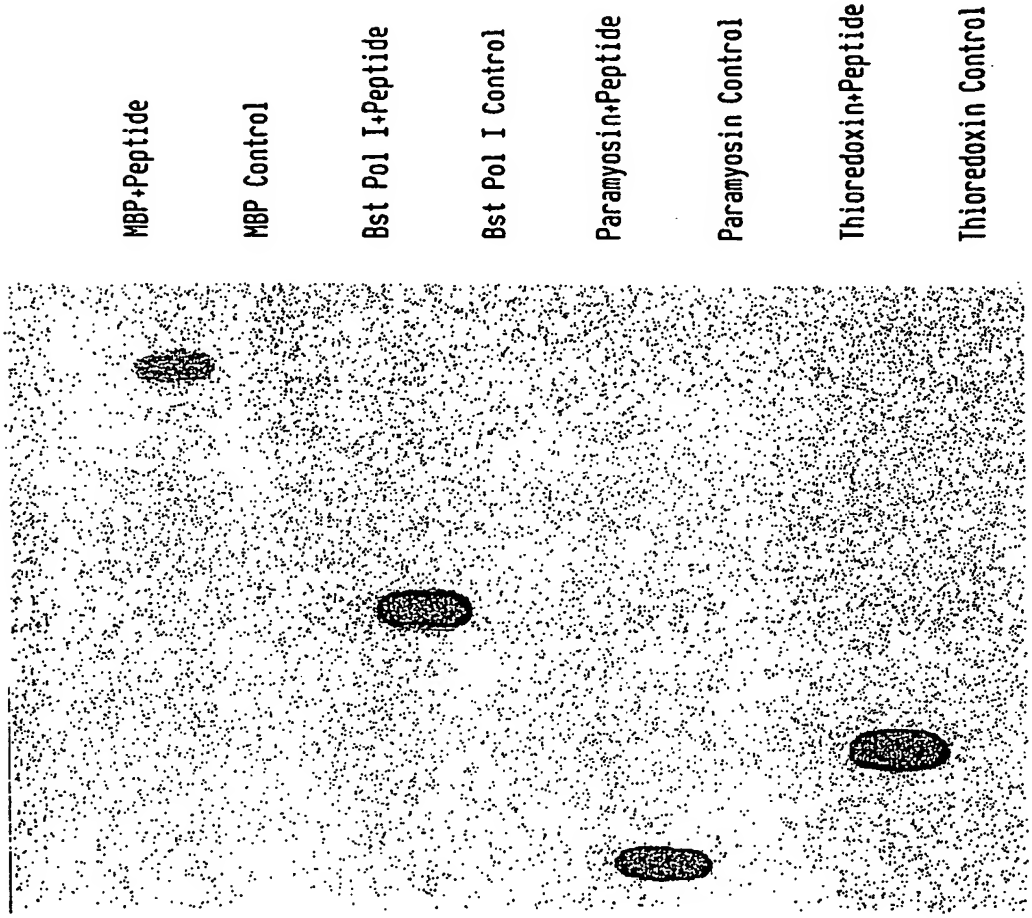


FIG. 7

WESTERN BLOTS OF PROTEINS LIGATED TO A BIOTINYLATED PEPTIDE



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22776

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 19/00; C12N 9/10, 15/62; C12P 21/02, 21/04
US CL :435/68.1, 69.7; 513/2; 530/402, 408; 536/23.4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/68.1, 69.7; 513/2; 530/402, 408; 536/23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,834,247 A (COMB et al) 10 November 1998, cols. 18-19, 40-45, 47-49, 75-77, and 87-89.	1-6, 9
X -- Y	CHONG, S. et al. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. Gene. 19 June 1997, Vol. 192, No. 2, pages 271-281, especially Figure 1B at page 273 and pages 274-277.	9 ---- 1-6, 10
X,P --- Y,P	EVANS, T.C. et al. Semisynthesis of cytotoxic proteins using a modified protein splicing element. Protein Science. 05 November 1998, Vol. 7, No. 11, pages 2256-2264, especially Figure 1B at page 2258 and pages 2257 and 2259-2261.	1-6, 9 ---- 10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
10 MARCH 2000	10 APR 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	WILLIAM W. MOORE
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

JOYCE BRIDGERS
PARALEGAL SPECIALIST
CHEMICAL MATRIX

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22776

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	MUIR, T.W. et al. Expressed protein ligation: A general method for protein engineering. Proceedings of the National Academy of Sciences, U.S.A. June 1998, Vol. 95, pages 6705-6710, see entire publication.	1-6, 9 ----- 7, 8, 10
X,P	EVANS, T.C. et al. The Cyclization and Polymerization of Bacterially Expressed Proteins Using Modified Self-splicing Inteins. The Journal of Biological Chemistry. June 1999, Vol 274. No. 26, pages 18359-18363, especially pages 118360-118362.	1-7, 9
Y	TELENTI, A. et al. The Mycobacterium xenopi GyrA Protein Splicing Element: Characterization of a Minimal Intein. Journal of Bacteriology. 14 October 1997, Vol. 179, No. 20, pages 6378-6382, especially pages 6379-6381.	8